Mechanism of the Irreversible Inhibition of Aspartate Aminotransferase by the Bacterial Toxin L-2-Amino-4-methoxy-trans-3-butenolic Acid*

ROBERT R. RANDO, NOEL RELYEA, AND LORNA CHENG

From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

The naturally occurring toxin L-2-amino-4-methoxy-trans-3-butenolic (AMB) acid irreversibly inhibits pyridoxal phosphate-linked aspartate aminotransferase. The inhibitor is a substrate for the enzyme, and as such is converted into a highly reactive intermediate which chemically reacts with an active site residue, thus irreversibly inactivating the enzyme. Enzymological and model studies on AMB are presented which enable one to determine the precise mechanism of action of this toxin. The mechanism involves Schiff base formation between the enzyme and toxin followed by α-C–H bond cleavage and aldime isomerization to generate a bifunctional Michael acceptor. This molecule alkylates an active site residue by an addition and elimination route.

EXPERIMENTAL PROCEDURE

Materials and Methods—Cytoplasmic, pig heart, aspartate aminotransferase (predominantly α subform) (3), α-ketoglutarate, L-aspartic acid, NADH, L-cysteine sulfinic acid, pyridoxal, pyridoxal phosphate, pyridoxamine, malic dehydrogenase, L-amino acid oxidase, and catalase were all products of the Sigma Chemical Co. The specific activity of the aspartate aminotransferase used was 285 units/mg, where 1 unit of enzyme will convert 1 μmole of aspartic acid to oxalacetate under standard conditions (4). The pure α subform of the enzyme is reported to have a specific activity of ~350 units/mg (5). AMB was a generous gift of Dr. J. Scannell of Hoffman-La Roche, Inc. Vinylglycine was prepared by the previously published procedure (6). KMB was prepared from AMB in the following way: a mixture of 4.2 mg (0.5 ml) of L-amino acid oxidase and 6 mg (0.2 ml) of catalase solution (1.5 x 10^4 units) were dialyzed against 1 liter of doubly distilled water overnight, 9.55 mg (0.073 mmol) of AMB were added to the dialyzed mixture, and the pH was adjusted to 7.2. In separate experiments it

* This work was supported by the National Institutes of Health Grants GM 20366 and NS 11550 and the Benevolent Foundation of Scottish Rite Freemasonry, Northern Jurisdiction, United States.

$Recipient of a United States Public Health Service Research Career Development Award GM 00014.

The abbreviations used are: AMB, L-2-amino-4-methoxy-trans-3-butenolic acid; KMB, 2-keto-4-methoxy-trans-3-butenolic acid.
was shown that AMB is an excellent substrate for the enzyme; somewhat better than 2-amino-5-butanone acid actually. Oxygen was bubbled through the reaction mixture at room temperature for 30 min. The reaction was stopped by passing the solution through a 8 ML ultrafiltration unit. The deproteinized filtrate was stirred with Dowex 50 H+ to remove any unreacted amino acid. The beads were removed by centrifugation and the supernatant (2.2 ml) was recovered containing 6.8 mg of the highly reactive ketoacid. This compound proved to be unstable to isolation and further purification in our hands. Fresh preparations of the ketoacid showed a λmax at 307 nm, log ε = 3.05 (apparent) in 0.1 M potassium phosphate, pH = 7.5. The effective λ is a likely lower limit since we don’t know what percentage of the keto acid is hydrated. It is likely to be high. This absorption can be completely wiped out by the addition of either sodium borohydride or mercaptoethanol in pH = 8.0. This is precisely as expected since the borohydride reduces the keto group to a hydroxy group and the mercapto reactions in a Michael fashion with the activated double bond.

Neither the reduced product (2-hydroxy-4-methoxy-3-butenoic acid) nor the Michael addition product was in the least as active as inhibitors of aspartate aminotransferase.

The sodium borohydride reduction experiments were conducted as follows: 5 characters (0.5 mmol) of KMB in 5 ml of methanol at 10°C was added slowly a 10-fold excess of sodium borohydride in 2 ml of cold methanol. The reaction was allowed to proceed for 20 min at 10°C and then 1 hour at room temperature. At the end of this time the solution was adjusted to pH 8.0 and the sodium borohydride solution was evaporated to near dryness and 2 ml of a saturated NaCl solution was added. This solution was continuously extracted with ether. The ether solution was dried over Na2SO4 and the ether was evaporated to afford 0.2 mg of a colorless oil which did not have an ultraviolet spectrum. The infrared and NMR spectra of this homogeneous material were entirely consistent with its assigned structure, 4-methoxy-3-hydroxy-3-butenoic acid. Furthermore, reduction of this compound in methanol took place with H2 being taken up with the concomitant formation of 4-methoxy-2-hydroxybutanoic acid. This molecule is identical in all respects with its independently synthesized counterpart, prepared by cyanohydration of 4-methoxypropionaldehyde followed by acid hydrolysis. The Michael addition reactions were run by dissolving 1 mmol of KMB in 1 ml of 0.1 M Tris, pH = 8.0. Ten millimeters of mercaptoethanol were added at room temperature. The solution was incubated for an additional 1/2 hour. The ultraviolet spectrum of the product of this reaction indicated a complete loss of starting material. This product formed a 2,4-dinitrophenylhydrazone derivative, which indicated that the keto group was still intact. The ultraviolet spectrum of the product of this compound was essentially identical with that of the 2,4-dinitrophenyl derivative of 3-ketobutanonate, indicating a lack of unsaturation in the molecule.

AMB-inhibited sample did not result in the loss of counts from the reduced protein. A likely lower limit since we don’t know what percentage of the keto acid is hydrated. It is likely to be high. The effective λ is a likely lower limit since we don’t know what percentage of the keto acid is hydrated. It is likely to be high. The absorption can be completely wiped out by the addition of either sodium borohydride or mercaptoethanol in pH = 8.0. This is precisely as expected since the borohydride reduces the keto group to a hydroxy group and the mercapto reactions in a Michael fashion with the activated double bond.

The activity of the enzyme was routinely assayed by the published procedure on a Gilford No. 240 recording spectrophotometer. Ultraviolet spectra were determined with a Cary 118 spectrophotometer. The enzyme was resolved into apoenzyme and cofactor by the method of Bentland and Kaplan (7). The pyridoxamine form of the enzyme was prepared by treating it with L-cysteine sulfinic acid according to the procedure of Jenkins and D’Ari (8).

Transamination reactions were similar to those described by Metzler and Snell (9). Incubations were done in 0.2 M acetate buffer, both in the presence and in the absence of 1 mM AI3+. Both pyridoxal and amino acid concentrations were generally 10 mM. Samples were mixed at 0°C, flushed throughly with N2, capped, and covered with aluminum foil before heating for various indicated times in a boiling water bath. Following incubation, samples were again stored at 0°C. Incubations using pyridoxal 5′-phosphate were done in 0.1 M phosphate buffer, pH 7, which was adjusted to 7.0 with CsCO3. All incubations contained 0.4 mM pyridoxal 5′-phosphate and 0.4 mM amino acid. The pH values of the solutions were determined at 25°C.

Electrophoretic separation of the components of the transamination reaction mixtures was achieved in a Durrum cell (Beckman Instrument, Inc., Palo Alto, CA). Running buffer, pH 1.8 (60:40:10 of formic acid:acetic acid:water) buffer, pH 3.1 (5 ml of pyridine + 100 ml of acetic acid per liter). Localization of pyridoxal derivatives was by fluorescence; amino acids, as well as pyridoxamine, were located by staining with ninhydrin.

Following purification by electrophoresis, pyridoxal and pyridoxamine concentrations were measured by elution into 0.1 M phosphate buffer, pH 6.7 and readings of the absorbance maxima at 315 and 325 nm, respectively. Pyridoxal was also determined directly on aliquots of the transamination mixtures by interaction with phenylhydrazine (10). α-Keto acid concentrations were measured directly in the absence of 1 mM AI3+. Both pyridoxal and amino acid concentrations were measured by ninhydrin (11). As shown in Fig. 1, the holoenzyme in the pyridoxal form is inhibited more slowly than the pyridoxamine form of the enzyme. Also inhibition of this latter form of the enzyme is competitively slowed down by the presence of substrate (aspartic acid is hydrated). It is likely to be high.
must be the inactivating agent and not some minor decomposition product of it. KMB itself has a $\lambda_{\text{max}}$ at 307 nm with a $\epsilon = 1.13 \times 10^4$ (log $\epsilon = 3.05$). Reduction with sodium borohydride or reaction with mercaptoethanol at pH = 8 collapses the spectrum and yields products that have no effect on the enzyme's activity. This is what would be predicted since the structure of the borohydride reaction product is $\text{CH}_2\text{O} - \text{CH} = \text{CH} - \text{CHOH} - \text{CO}_2\text{H}$ and Michael adduct with mercaptoethanol is presumed to be $\text{O} - \left(\text{HO-CH}_2\text{CH}_2\text{S}_{\text{al}}\text{CH}_2\text{CH}_2\text{CO}_2\text{H}\right.$.

Neither of these molecules would be expected to react with the enzyme.

**Identification of Cofactor after AMB-induced Inactivation of Enzyme**—In these experiments, two identical samples of enzyme were prepared. The first was treated with AMB and the second with L-cysteine sulfonic acid, a reagent which quantitatively converts the pyridoxal form of the enzyme to the pyridoxamine form (8). After treatment with these reagents both samples were resolved and the structure of the cofactors was determined to be identical after purification by paper electrophoresis (7). These experiments are also consistent with the intermediacy of 7 as the actual inhibitor since its resolution would yield pyridoxamine phosphate.

**Model Studies on Interaction of AMB and Vinylglycine with Pyridoxal**—The model systems developed by Snell for studying vitamin B$_6$ cofactored transaminations involves heating mixtures of pyridoxal, the amino acid, and a metal ion such as aluminum at pH = 5. Under these conditions amino acids such as glutamate and aspartate are equilibrated with their $\alpha$-keto analogues (9), whereas amino acids with leaving groups attached to their $\beta$ carbon atoms, such as serine or cysteine, undergo elimination reactions (13).

In the initial experiments (Fig. 2A), AMB, pyridoxal, and aluminum ions were heated together at 100$^\circ$ in pH = 4.8 ammonium ion buffer. If the concentrations of AMB and pyridoxal decrease concomitantly with an increase in pyridoxamine formation then we can be sure that transamination is occurring. On the other hand, if pyridoxal is not lost but AMB is, and no pyridoxamine is formed, then isomerization must be occurring. In Fig. 2A it can be seen that virtually all of the AMB and pyridoxal lost can be accounted for by the formation of pyridoxamine. Therefore, transamination can be accounted for virtually all of the loss of AMB. Note that after a time a fall-off in pyridoxamine concentration occurs. This can be accounted for by the formation of several new fluorescent compounds as judged by paper electrophoresis. This could represent further reaction of the pyridoxamine with a decomposition product of KMB. At 60$^\circ$ there is no fall-off but instead a smooth, albeit slower, rise of pyridoxamine concomitant with a loss of AMB and pyridoxal (Fig 2B). Thus, little or no isomerization is occurring, otherwise pyridoxamine would not be formed. Therefore, these experiments mirror the enzymological studies with AMB—in both cases transamination predominates. The same results in the model system are obtained when pyridoxal phosphate is substituted for pyridoxal.

It is of interest to compare the above results with those obtained with vinylglycine in the Snell model system. In the presence of aluminum ions and pyridoxal, vinylglycine yields mostly $\alpha$-ketobutyrate as can be seen from Fig. 3A; little pyridoxal is lost and little pyridoxamine is formed. The
Irreversible Inhibition of Aspartate Aminotransferase

Fig. 2. A, model studies—the transamination of AMB by pyridoxal (Al3+) at 100°. The transamination studies were run as described under “Materials and Methods.” AMB (10 μmol) was heated with 10 μmol of pyridoxal and 1 μmol of aluminum sulfate in the ammonium acetate buffer. At various times aliquots were removed and assayed for pyridoxamine, pyridoxal, and AMB concentrations as described. No attempt was made to analyze for keto acid due to its known instability. In the figure shown above (O) refers to pyridoxamine concentrations, (●) to pyridoxal, and (▲) to AMB concentrations. As time progresses it can be seen that the pyridoxamine level peaks and then decreases. This decrease in pyridoxamine levels occurs simultaneously with the appearance of several new fluorescent peaks as analyzed by paper electrophoresis. We assume that these new compounds are reaction products of KMB (or decomposition products thereof) with pyridoxamine. B, the transamination of AMB by pyridoxal (Al3+) at 60°. The experiments were identical with those shown in Fig. 2A except they were conducted at 60°. (O) refers to the pyridoxamine concentrations, (▲) to the pyridoxal concentration, and (●) to the AMB concentration.

Fig. 3. A, model studies—the transamination and isomerization of vinylglycine by pyridoxal (Al3+) at 100°. Using the system already described, 10 μmol of vinylglycine was heated with 10 μmol of aluminum sulfate. The ketoacid produced was analyzed during this reaction and proved to be α-ketobutyrate. In the graph shown above vinylglycine is lost and can be accounted for almost completely by the formation of α-ketobutyrate. This means that isomerization predominates over transamination with vinylglycine, a result exactly opposite to that obtained with AMB. In the absence of aluminum ions, however, transamination predominates (Fig. 3B).

Reduction of AMB-inactivated Enzyme by Sodium Borohydride—Two identical samples of enzyme in the aldehyde form were made up. The first of these two was treated with L-cysteine sulfinate and dialyzed (Fig. 4a). The second was treated with AMB and also dialyzed (Fig. 4c). The AMB-inactivated enzyme was treated with sodium borohydride which immediately collapsed its spectrum into that of Fig. 4b. The borohydride-reduced enzyme was not susceptible to resolution.

The above experiment was repeated with NaB3H, the results of this experiment are shown in Table 1. As can be seen the amount of tritium incorporated into the AMB inactivated enzyme is equal to the amount incorporated into an equal quantity of the pyridoxal enzyme. Denaturation of these labeled proteins did not lead to a loss of incorporated radioactivity. Unfortunately, the pyridoxamine enzyme also incorporates counts. This is not unusual, however, for borotritide will nonspecifically incorporate tritium into polypeptide backbones (14).

DISCUSSION

The sodium borohydride experiments described above are essential for the delineation of the steps in the inactivation process subsequent to the formation of I by the normal transamination process. Following its formation, the highly reactive Michael acceptor I must then undergo a chemical reaction with a nucleophilic amino acid residue to account for the irreversible inhibition observed. One could postulate a direct Michael addition as shown below,
Irreversible Inhibition of Aspartate Aminotransferase

Fig. 4. The sodium borohydride reduction of AMB-inactivated aspartate aminotransferase. Aspartate aminotransferase (1.54 mg, 0.027 μmol of active subunit) in the pyridoxal form was dissolved in 1.2 ml of 0.1 M potassium phosphate buffer (pH 7.0). Two identical samples were made up in this manner. The first sample, shown in graph a, was treated with 20 mM L-cysteine sulfinate acid according to the published procedure (8). The second sample was treated with AMB and completely inhibited c. Both samples were extensively dialyzed against the phosphate buffer. The cysteine sulfinate-treated sample is shown in graph a and the AMB-inactivated sample in graph c. The AMB-inactivated enzyme has a λmax at 347 nm, ε = 28,500, log ε = 4.46. The sample from graph c was then treated with 10 μmol of sodium borohydride for 0.5 hour in ice and dialyzed against the phosphate buffer. The spectrum of this material is shown in graph b. Now importantly, under the published resolution conditions the cysteine sulfinate-treated material (a) is clearly resolved after 8 hours of resolution (7). Even after 40 hours no resolution is obtained with the borohydride-reduced complex (b). As precipitation of the protein becomes a problem, the resolution cannot be run for too much longer than 40 hours. However, even when the resolution procedure was continued to 3 days no resolution of the still soluble protein had occurred. Furthermore, precipitation of the protein with trichloroacetic acid did not lead to the liberation of any pyridoxamine in the supernatant.

Table I

<table>
<thead>
<tr>
<th>Incorporation of tritium into borotritide-reduced AMB-inactiuated enzyme</th>
<th>Pyridoxal enzyme</th>
<th>AMB inac-</th>
<th>Pyridoxamine enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>net dpm (× 10^3)</td>
<td>5150</td>
<td>5475</td>
<td>2235</td>
</tr>
</tbody>
</table>

where B is an electronegative atom—O, S, or N. However, there are several problems with this interpretation. First of all, 4 could not account for the ultraviolet spectrum of the AMB-inactivated enzyme. The ultraviolet spectrum of 4 above 300 nm should be that of a pyridoxamine derivative. Secondly, in the vinylglycine-induced irreversible inhibition of the enzyme, a structure very much like 4 represents the inactive enzymes (H substituted for OCH3) (9). See 5 below.

In this case, the λmax of the inactivated enzyme 5 is indistinguishable from that of the pyridoxamine enzyme. Furthermore, there are certain chemical difficulties with 4. Two electronegative atoms are attached to the same carbon atom (B and O). This is known to represent a relatively unstable constellation of atoms. However, if methanol were eliminated from compound 4 we arrive at the structure of what we believe to be the actual inhibited enzyme 6. If structure 6 represents the inactivated enzyme, a definite prediction should be realized.

Sodium borohydride should collapse the λmax at 350 nm of the AMB-inhibited enzyme triplet to a spectrum that is identical with that of the pyridoxamine form of the enzyme. Sodium borohydride has been often used as a reducing agent for the aldimine linkage in pyridoxal phosphate-linked enzymes (15, 16). Furthermore, the borohydride-reduced enzyme should no longer be resolvable since the cofactor is now covalently bound to the enzyme 7.
Irreversible Inhibition of Aspartate Aminotransferase

These predictions have been realized as shown in Fig. 4. It is also clear that the reduction of 6 by sodium borotritide should lead to the incorporation of tritium into the enzyme in the same amount as is found in the reduction of the pyridoxal form of native enzyme. This expectation was also realized.

In summary, we contend that the AMB-induced irreversible inhibition of aspartate aminotransferase involves the following sequence of steps:

We conclude that covalent bond formation has occurred between AMB and an active site residue(s) based on the following experiments: (a) when the inactivated enzyme is resolved of its cofactor, enzymatic activity cannot be regained by adding back fresh pyridoxal phosphate. (b) sodium borotritide reduction of the inactivated enzyme covalently fixes the cofactor to the protein backbone and results in the incorporation of tritium in the protein, and (c) the closely analogous inactivation of the enzyme by [%]vinylglycine results in the incorporation of 1 mol of inhibitor/m01 of subunit.3 Our conclusion that Step 2 occurs is based on the following kinds of evidence: (a) the cofactor in the inactivated enzyme is pyridoxamine phosphate, (b) the pyridoxamine form of the enzyme is rapidly inactivated by KMB relative to the pyridoxal phosphate form of the enzyme and the apoenzyme, and (c) model studies with AMB and pyridoxal show that the transamination process is highly favored over isomerization. The conclusions based on these experiments are, of course, consistent with more indirect kinds of observations. For example, the ultraviolet spectral shift that occurs when the enzyme is inhibited by AMB is not reconcilable with an isomerization route, which would have left the pyridoxal moiety intact. Furthermore the ultraviolet spectrum of the AMB-inhibited enzyme is the same between pH values 4 and 9. This observation is also not consistent with the presence of a pyridoxal enzyme.

Following the formation of the highly reactive 5, a reaction occurs between an active site nucleophile and this intermediate to generate the inactive enzyme 6. In this scheme, 6 is responsible for the ultraviolet absorption above 300 nm of the inhibited enzyme. The addition-elimination reaction postulated here is a well known process in organic chemistry, and is, in fact, the pathway by which most displacement reactions occur at vinyl carbon atoms. Since the methoxyl group is not a very good leaving group it might not be expected to be displaced very often. However, the intermediate 7 would partition to give the virtual product 5 and 6 but the formation of the latter is irreversible as a consequence of the loss of methanol into solution. So, indeed, 6 is a sink into which all of 5 must eventually fall.

Our conclusion that 6 is the chromophore of the inhibited enzyme is based primarily on the borohydride reduction experiments. As previously mentioned this reagent has been used generally to reduce the aldimine linkage in pyridoxal containing enzymes (15, 16). If 6 is a true representative of the inhibited enzyme then its reduction should (a) collapse the triplet at 350 nm to a spectrum characteristic of a pyridoxamine derivative and (b) chemically fix the cofactor to the enzyme. Both of these expectations have been realized. Furthermore, since we already know that the cofactor in the inhibited enzyme is in the pyridoxamine form, the fact of nonresolvability after borohydride reduction cannot mean that we are simply reducing a Schiff base between an ε-amino group of a lysine and pyridoxal phosphate.

Finally when the reduction of the AMB-inhibited enzyme is carried out with borotritide we find the tritium is incorporated into the reduced enzyme in the same amounts as it is incorporated into the pyridoxal form of the holoenzyme.

In the scheme presented, the highly absorbing peak centered at 347 nm in the AMB-inactivated enzyme must be due to the chromophore.

Certainly, the pyridine spectrum is hidden underneath this highly absorbing triplet. It is not possible to go to the literature and find spectral correlates with model systems, because we don’t know what B is. However, some model systems in the enamine series are worth considering. For example,

has a λmax at 308 nm and an ε = 40,000 (log ε = 4.60) and

*Unpublished experiments and Ref. 6.
Irreversible Inhibition of Aspartate Aminotransferase

has a $\lambda_{\text{max}}$ at 322 nm and an $\varepsilon = 39,500$ (log $\varepsilon = 4.59$) (17, 18). The $\lambda_{\text{max}}$ of the AMB inhibited enzyme is at 347 nm with an $\varepsilon = 28,500$ (log $\varepsilon = 4.46$). It is clear that this data also is supportive of the assertion that 6 is the chromophore of the inactivated enzyme. It is interesting to note that not only the position of $\lambda_{\text{max}}$ but also the $\varepsilon$ is as expected. The borohydride reduction of 6 led to a pyridoxamine spectrum which clearly had a smaller $\varepsilon$ than the chromophore of 6. At pH = 7.0 pyridoxamine has a $\lambda_{\text{max}}$ at 325 nm and $\varepsilon = 7,700$ (log $\varepsilon = 3.88$) (19). Certainly B might be a nitrogen in our case. In the irreversible inhibition of aspartate aminotransferase by the activated nitrogen mustard, $\beta$-chloroalanine, an active site lysine is alkylated (20). The same lysine might be alkylated in the example reported here. Experiments are in progress to decide this point.

As noted under “Results” the model studies with AMB closely parallel the first steps in the inactivation process, with transamination predominant. By way of comparison, vinylglycine is almost exclusively isomerized to afford $\alpha$-ketobutyrate in the presence of pyridoxal and $\text{Al}^{3+}$. A possible mechanism of this isomerization is shown below:

On the other hand, when $\text{Al}^{3+}$ is omitted from the reaction mixture, the ratio shifts and transamination occurs almost exclusively (Fig. 3B). A possible function of the aluminum ion may be to stabilize the developing carbanion character on the $\gamma$ carbon 3 of vinylglycine.

The fact that AMB and vinylglycine undergo different reactions in the presence of $\text{Al}^{3+}$ is probably a reflection of the stabilizing effect on the double bond by the electron-donating methoxyl group in AMB. Transamination predominates with AMB in the model studies simply because in this way the conjugative interaction between the methoxyl oxygen and the double bond is not lost.

By now, AMB and vinylglycine have been tested as substrates with two other enzymes. Both sheep liver threonine dehydratase and pyridoxal phosphate-linked tryptophan synthetase utilize vinylglycine as a substrate and isomerize it to $\alpha$-ketobutyrate (21, 22). Interestingly enough, however, AMB appears to be an irreversible inhibitor of the tryptophan synthetase (22). This suggests that isomerization does not readily occur in this system either, and that the mechanism of the inactivation may be the same as that reported here.

REFERENCES

Mechanism of the irreversible inhibition of aspartate aminotransferase by the bacterial toxin L-2-amino-4-methoxy-trans-3-butenoic acid.
R R Rando, N Relyea and L Cheng


Access the most updated version of this article at http://www.jbc.org/content/251/11/3306

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/11/3306.full.html#ref-list-1